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PRINCIPAL INVESTIGATOR: Jacob Arakkal-Nellissery, Ph.D.

CONTRACTING ORGANIZATION: Temple University School of Medicine
Philadelphia, Pennsylvania 19140

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Jacob Arakkal-Nellissery, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Temple University School of Medicine
Philadelphia, Pennsylvania 19140

E-Mail: jarakkal@unix.temple.edu

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INTRODUCTION

Breast cancer is a major health problem affecting American women, accounting for 181200 new cases diagnosed in 1997 and 17% of all cancer deaths (Parker et al, 1997). Among the various epidemiological factors that contribute to the development of breast cancer, a positive family history of breast cancer in a first degree relative is associated with a doubling of risk (Claus et al, 1990). It has been estimated that genetically inherited forms of breast cancer account for approximately 5%-10% of all breast cancer cases (Weber and Garber, 1997). Genetic linkage analysis in large kindred's with several affected individuals, has localized two breast cancer susceptibility genes, Brca1 (Hall et al, 1990) and Brca2 (Wooster et al, 1994) to the long arm of chromosomes 17q21 and 13q 12 respectively. These two genes have recently been isolated by positional cloning strategies (Miki et al, 1994; Wooster et al, 1995). Germline mutations in Brca1 predispose the carrier females to early onset breast and ovarian cancer, while Brca2 mutations increase the susceptibility to breast and pancreatic cancer. Unlike Brca1, mutations in Brca2 also increase the risk of male breast cancer (Stratton et al, 1994). Identification of these 2 breast cancer predisposing genes has enabled the development of diagnostic tools for carrier detection and therapeutic intervention in familial breast cancer.

Brca2 encodes a protein of 3418 amino acids with no significant sequence similarity to Brca1or any other known protein. A portion of BRCA2 protein near the N-terminus encoded by exon 3 has sequence similarity to the transcription activation domain of c-jun. Gene fusion experiments including exon 3 of BRCA2 have revealed stimulation of transcription in yeast and mammalian cells (Milner and Ponder, 1997). BRCA2 gene knockout experiments in mice revealed embryonic lethality and developmental arrest due to a defect in cell proliferation (Sharan et al, 1997). Using a yeast two hybrid screen, these authors also demonstrated an association of Brca2 with the DNA repair protein, Rad51. The involvement of Brca2 in the Rad51- dependent double strand break repair pathway correlates well with the observation of increased radiation sensitivity of Brca2-/- embryos in vivo and with hypersensitivity to genotoxic agents in vitro of cultured cells derived from truncated Brca2 mutant embryos (Patel et al, 1998). The precise timing of cell cycle arrest in G1 and G1/M phases with a concomitant increase in the expression of p53 and p21 which signaled DNA damage were postulated to define a role for Brca2 in DNA repair (Patel et al, 1998).

Even though, the above data based on knockout mouse models (Ludwig et al, 1997; Suzuki et al, 1997) explain a plausible function of Brca2 in development, it does not fully address the tumor suppressor role for this gene, the loss of function of which in tumor cells ultimately leads to increased cellular proliferation. Blocking the expression of Brca2 in differentiated mammary epithelial cells and in breast cancer cell lines using antisense technology to study the resulting phenotype in terms of any alteration in the levels of cell cycle control proteins will be crucial to an understanding of this mammary carcinogenesis pathway and in identifying the key mediators of Brca2 function. Complementary experiments aimed at reversing the malignant phenotype of breast cancer cell lines by introduction of the wild type gene and assaying for its potential to cause tumors in vivo would, besides attesting the role of Brca2 as tumor suppressor, provide a rationale for the development of corrective therapies for specific types of breast cancer. This is particularly important since both radiation and many cytotoxic drugs used currently in the treatment of cancer cause DNA damage and subsequent arrest of cell division, accounting in part for their efficacy. Hence an understanding of Brca2 mechanism could play a decisive role in breast cancer treatment.

This project is aimed at addressing the role of Brca2 gene as a tumor suppressor in *in vitro* and *in vivo* assays. As a first step in realizing the aims of this project, I have isolated and cloned copies of the full length Brca2 cDNA and obtained preliminary data on its expression pattern in normal and breast cancer cell lines. I have successfully used RT-PCR to synthesize full length Brca2 cDNAs from a normal breast epithelial cell line, MCF10A. This cDNA has been cloned into a mammalian expression vector, pBKCMV in both the sense and antisense orientations.

An second antisense construct encompassing the 5' untranslated region (UTR) plus exons 1-7 of Brca2 gene was designed and cloned in ptTAS, a tetracycline inducible mammalian expression vector. This construct besides providing a dose dependent expression of antisense RNA can also be used in rescue experiments in which restoration of Brca2 function will be tested by the introduction of plasmid containing only the coding sequence. The plasmid DNAs have already been transfected into normal breast epithelial and breast cancer cell lines

Four cancer cell lines with defective BRCA2 gene were identified and the mutations confirmed by SSCP analysis and sequencing of PCR amplified exon DNAs. Stable cell lines expressing sense/antisense BRCA2 protein were generated and analyzed by Western blotting experiments. These are being characterized with respect to cellular proliferation and morphology. using *in vitro* assays.

EXPERIMENTS AND RESULTS

1. Construction of full length Brca2 cDNA:

2.

Total RNA was isolated from MCF10A cells grown to 70% confluency, using RNAzol method. Poly A(+) RNA was purified using oligotex beads (Qiagen). Total RNA (10µg) or 500ng of polyA(+) RNA was used as template for first strand cDNA synthesis with oligo dT primer and Superscript (Gibco-BRL) reverse transcriptase. The reaction was performed at 48°C to prevent the formation of secondary structure and to facilitate synthesis of longer full length cDNA. An aliquot (5µl) of this cDNA was used in LA-PCR using Brca2 gene-specific primers and Expand High Fidelity Taq polymerase mixture (Boehringer Mannheim

LA-PCR was performed on 5µl of the first strand cDNA sample with primers 1F and 3R using Expand High Fidelity PCR system (Boehringer Mannheim). An alternate forward primer, brex1F (5'AGATCTGTGGCGCGAGCTTCTGAAACTAG), designed to include 5' untranslated region of the Brca2 mRNA was also used along with the reverse primer (3R) in a separate PCR reaction. The reaction mixture was essentially identical to the one described above. The amplification conditions, however, were chosen to facilitate longer extension times as follows:

Initial denaturation at 94°C, 2min followed by 10cycles of
Denaturation at 94°C, 15s
Annealing at 62°C, 30s
Elongation at 68°C, 8min followed by 20 cycles of
Denaturation at 94°C, 15s
Annealing at 62°C, 30s
Elongation at 68°C, 8min + an autoextension of 10s for each cycle
and a final elongation cycle at 72°C for 7 min.

The gel-purified DNA was blunt ended and cloned first into pBluescript and subsequently into the mammalian expression vector, pBKCMV. Cloning in the EcoRV site of pBluescript facilitated a wider choice of restriction sites on either side for subsequent directional cloning into the expression vectors. The authenticity of this clone (pBrcfl-6) was verified by sequencing the ends of the insert DNA using T3 and T7 primers and by restriction digestion analysis. This full length Brca2 clone is being further characterized with respect to its sequence integrity and parallel experiments are in progress to introduce an expression

construct of this clone into breast cancer cell lines MCF7 and MDA-MB468 in order to obtain stable constitutive expression of Brca2 protein.

2. Construction of 5' antisense Brca2 expression plasmid in a Tet-inducible vector, ptTAS:

The recent development of tetracycline-regulated, inducible expression systems have enabled precise and reversible control of exogenous gene regulation in mammalian cells (Shockett and Schatz, 1996). Tet-On and Tet-Off systems offer the advantage of precise temporal and quantitative control of gene expression by simply varying the concentration tetracycline or its derivative doxacycline in the medium, in a dose-dependent manner

A modified Tet-Off vector incorporating tTA and TRE in the same plasmid, ptTAS (gift from Dr. Clement Lee) was used to construct a 5' Brca2 antisense expression vector. This vector was chosen since it required only one round of transfection to yield a stable cell line. The cDNA corresponding to exons 1-7 of Brca2 gene was amplified by RT-PCR using a nested set of reverse (brex10R and brex7R) primers and brex1F as the forward primer. The oligonucleotide sequences are :

Brex10R: 5' GGCATTGACTTTCCAATGTGGTC

Brex7R: 5' ACTGAATTCCAGGATCCACCTCAGCTCCTAG

The product was gel purified and digested with EcoRI to generate one cohesive end and ligated to EcoRI/BamHI double digested ptTAS plasmid DNA. The antisense orientation of the insert DNA was confirmed by PCR using CMV promoter primer and Brca2 specific primers. One of the positive clones, pTBrcut-1 was sequenced for additional confirmation. This DNA along with a another cotransfection plasmid containing a selectable marker will be electroporated into MCF10A cells to obtain a stable cell line expressing Brca2 antisense transcript under tetracycline control.

3. Identification of BRCA2 deficient Cell lines:

Oligonucleotide primers corresponding to exons 1-27 of the BRCA2 gene were synthesized based on the published sequence information. These primers were used to amplify genomic DNA isolated from ten different cancer cell lines. The PCR amplified products were subjected to SSCP -gel analysis and the ones that showed mobility differences were sequenced to identify the mutation. Using this protocol we selected 4 cell lines in which BRCA2 gene was defective. Western blot of cell lysates using commercially available antibodies was used to confirm the absence of a functional BRCA2 protein in one of the cell lines.

4. Expression of BRCA2 in Normal/ Mutant Cell lines:

The full length BRCA2 cDNA clone was transfected into a -/- cell line (Capan-1) using Lipofectamine Plus reagent. Stable clones were selected using G418 and expanded. Production of the full length protein in this clone was monitored by Western blotting experiments. The growth characteristics of this clone are being assayed.

The antisense BRCA2 plasmid was transfected into a normal breast epithelial cell line, MCF-10A. However, none of the stable clones showed a dose-dependent induction of the antisense transcript. A newly designed construct engineered using Ecdysone- Inducible expression system will be used in future experiments.

SUMMARY AND CONCLUSIONS

The proposed aims of this project were : 1. to obtain a full length Brca2 cDNA through a variety of strategies and clone it in a mammalian expression vector. 2. To introduce Brca2 expression plasmids into normal and breast cancer cell lines and obtain stable clones. 3. To study the phenotype of cells expressing the sense and antisense RNA by in vitro assays and 4. To test the tumorigenic potential of the transfected cells in vivo using the nude mouse model.

This report details the accomplishment of tasks 1-3 of the first technical objective, namely , constructing a full length cDNA for Brca2 gene and cloning it in an expression vector. The extremely high size of Brca2 mRNA posed some initial problems in isolating a full length cDNA clone by library screening.

Smaller clones corresponding to portions of the Brca2 gene were isolated from a jurkat cDNA library. However these short fragments were incontiguous and hence could not be assembled to generate the entire Brca2 cDNA. Construction of two full length clones (differing in the extent of 5' untranslated sequences) was accomplished by RT- PCR using sets of Brca2 specific primers and the LA-PCR system. The complete cDNAs were cloned in a mammalian expression vector, pBKCMV and characterized by restriction digestion analysis and partial sequencing. An antisense expression plasmid encompassing Brca2 exons 1-7 was designed and engineered in the tetracycline regulated expression system, ptTAS. Four cancer cell lines with mutated BRCA2 genes were identified. Stable cell lines expressing sense/antisenseBrca2 gene product have been generated. Preliminary data on the expression profile of Brca2 mRNA in normal and breast cancer cell lines have been obtained. In conclusion, I have generated full length and antisense Brca2 cDNA, engineered it in expression plasmids, introduced into normal and breast cancer cell lines. Experiments are in progress to test the activity of these modified cells *in vivo* using a nude mouse model.

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